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J. Billingsley

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AUSTRALIA

Patents Act 1990

Garvan Institute of Medical Research

PROVISIONAL SPECIFICATION

Invention Title:

Novel translocation assay

The invention is described in the following statement:

Field of the invention

The present invention relates to a novel *in vitro* assay for determining the amount of a protein, in particular a membrane transport protein, that is translocated to a plasma membrane of a cell. In one embodiment, the present invention provides a method of identifying an agent that modulates the translocation of a protein, in particular a membrane transport protein to a plasma membrane and the activity of that protein.

Background of the invention

General Information

As used herein the term "derived from" shall be taken to indicate that a specified integer may be obtained from a particular source albeit not necessarily directly from that source.

Unless the context requires otherwise or specifically stated to the contrary, integers, steps, or elements of the invention recited herein as singular integers, steps or elements clearly encompass both singular and plural forms of the recited integers, steps or elements.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated step or element or integer or group of steps or elements or integers but not the exclusion of any other step or element or integer or group of elements or integers.

The embodiments of the invention described herein with respect to any single embodiment shall be taken to apply *mutatis mutandis* to any other embodiment of the invention described herein.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

The present invention is not to be limited in scope by the specific examples described herein. Functionally-equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

All the references cited in this application are specifically incorporated by reference herein.

The present invention is performed without undue experimentation using, unless otherwise indicated, conventional techniques of molecular biology, microbiology, virology, recombinant DNA technology, peptide synthesis in solution, solid phase peptide synthesis, and immunology. Such procedures are described, for example, in the following texts that are incorporated by reference:

Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, New York, Second Edition (1989), whole of Vols I, II, and III;

DNA Cloning: A Practical Approach, Vols. I and II (D. N. Glover, ed., 1985), IRL Press, Oxford, whole of text;

Oligonucleotide Synthesis: A Practical Approach (M. J. Gait, ed., 1984) IRL Press, Oxford, whole of text, and particularly the papers therein by Gait, pp1-22; Atkinson *et al.*, pp35-81; Sproat *et al.*, pp 83-115; and Wu *et al.*, pp 135-151;

Nucleic Acid Hybridization: A Practical Approach (B. D. Hames & S. J. Higgins, eds., 1985) IRL Press, Oxford, whole of text;

Immobilized Cells and Enzymes: A Practical Approach (1986) IRL Press, Oxford, whole of text;

Perbal, B., A Practical Guide to Molecular Cloning (1984);

Methods In Enzymology (S. Colowick and N. Kaplan, eds., Academic Press, Inc.), whole of series;

J.F. Ramalho Ortigão, "The Chemistry of Peptide Synthesis" *In:* Knowledge database of Access to Virtual Laboratory website (Interactiva, Germany);

Sakakibara, D., Teichman, J., Lien, E. Land Fenichel, R.L. (1976). *Biochem. Biophys. Res. Commun.* 73 336-342

Merrifield, R.B. (1963). *J. Am. Chem. Soc.* 85, 2149-2154.

Barany, G. and Merrifield, R.B. (1979) in *The Peptides* (Gross, E. and Meienhofer, J. eds.), vol. 2, pp. 1-284, Academic Press, New York.

Wünsch, E., ed. (1974) *Synthese von Peptiden in Houben-Weyls Metoden der Organischen Chemie* (Müler, E., ed.), vol. 15, 4th edn., Parts 1 and 2, Thieme, Stuttgart.

Bodanszky, M. (1984) *Principles of Peptide Synthesis*, Springer-Verlag, Heidelberg.
Bodanszky, M. & Bodanszky, A. (1984) *The Practice of Peptide Synthesis, Springer-Verlag,* Heidelberg.
Bodanszky, M. (1985) *Int. J. Peptide Protein Res.* 25, 449-474.
Handbook of Experimental Immunology, Vols. I-IV (D. M. Weir and C. C. Blackwell, eds., 1986, Blackwell Scientific Publications).

Description of the related art

One of the most important activities performed by any cell is the transport of materials across the plasma membrane. This activity is essential for the survival of all organisms, from simple unicellular organisms, eg bacteria, to complex multicellular organisms, eg humans. Not only does membrane transport facilitate the uptake of, for example, nutrients and ions, but also the excretion of waste products, and the secretion of signalling molecules.

The process of membrane transport itself is performed by a large class of proteins known as "transporters" or "membrane transporters" or "membrane transport proteins". These proteins may form protein channels in the plasma membrane. This class include a vast number of proteins that are only related in their ability to transport other molecules across a cell membrane. It is hypothesized that the number of proteins involved in membrane transport constitute approximately 5% to 10% of known open reading frames in most sequenced genomes. Membrane transport proteins are generally localised both intracellularly and within the plasma membrane, however, as the membrane-localised form is significant for transport activity, the amount of any membrane transport protein present in the plasma membrane may affect the transport of substrates (both naturally-occuring substrates and small molecules) into and/or outside of the cell. Exemplary membrane transport proteins include the glucose-transporters (e.g. GLUT1, GLUT4) and ion transporters that transport $C1^-$, K^+, Na^+ or $S0_4^{2-}$ ions, amongst others (e.g. cystic fibrosis transmembrane regulator (CFTR), pendrin, human ether-a-go-go (HERG)). As will be known to those skilled in the art, membrane transport proteins may function in the transport of multiple substrates, such as, for example, in the same direction (eg., symport) or in the opposite direction (eg., antiport) across the plasma membrane.

Cells utilize a wide range of transport mechanisms, all of which are controlled by transport proteins.

Facilitated diffusion utilizes membrane protein channels to allow charged molecules (which otherwise could not diffuse across the cell membrane) to freely move across the membrane. For example, K+, Na+, and Cl- are transported across a cell membrane by such membrane protein channels.

Facilitative transport molecules convey molecules, such as, for example, sugars down a concentration gradient, i.e. from a region of high concentration of that molecule to a region of low concentration, in a process that does not require energy.

Active transport requires the expenditure of energy to transport the molecule across the membrane. Similar to facilitated transport, active transport is only limited by the number of membrane transport proteins present at the membrane.

Active, or coupled, membrane transporters transport substrates against a concentration gradient in a process that either requires energy expenditure or the use of another concentration gradient. For example, sodium dependent glucose transporters couple the transport of one molecule of glucose to two molecules of sodium. Sodium ions are transported down their concentration in a process that generates sufficient free energy to transport glucose against its concentration gradient allowing for concentration of glucose inside the cell to quite high levels.

As membrane transport proteins are involved in such a variety of functions that are essential to the survival of an organism, it is not surprising that several of these proteins have been found to be associated with disease in humans. For example, several forms of hearing loss in humans are associated with mutations in genes encoding transport proteins such as, for example, connexin 26, and pendrin, a proposed sulfate transporter. Defects in ion transporters are associated with a predisposition to cardiac arrhythmia, Menkes disease, Wilson's Disease, familial generalized epilepsy, benign infantile epilepsy, spinocerebellar ataxia and familial hemiplegic migraine amongst many others.

In addition to mutations that directly affect the activity of a protein, any defect that inhibits the trafficking of the relevant membrane transport protein to the correct subcellular location has also been shown to be linked with human disease. For example, it has been suggested that the membrane transport protein GLUT4 is abnormally localized in type II diabetes (Bryant *et al, Nature Reviews, Molecular Cell*

Biology, 3, 267-277, 2002). In the absence of insulin stimulation, localization of GLUT4, which transports glucose across the plasma membrane, is almost entirely intracellular. Upon the addition of insulin, GLUT4 translocates to the plasma membrane. Defects in this insulin stimulated GLUT4 translocation have been shown to be associated with insulin-resistance characteristic of type II diabetes mellitus (Kelley *et al, J. Clin. Invest. 97*, 2705-2713, 1996).

The most common mutations in the cystic fibrosis transmembrane regulator (CFTR) gene associated with cystic fibrosis (the ΔF508 mutation, Δ1507 mutation, K464M mutation, F508R mutation, and S5491 mutation, accounting for approximately 70% of CF patients) have been suggested to result in abnormal localization of the CFTR protein to the endoplasmic reticulum, where it is subsequently degraded (Cheng *et al, Cell, 63(4)*, 827-834, 1990). Such mutant forms of the CFTR protein have been observed to be localized at the apical region of the cytosol of cells, rather than within the plasma membrane. As the CFTR protein is a chloride channel, the reduction in the amount of this channel in the membrane results in a reduction in the movement of both sodium and water into the cell. The mislocalization of the CFTR protein has been suggested as a possible causative factor in the reduced movement of sodium and water observed in the lungs and intestines of subjects suffering from cystic fibrosis.

In the case of cardiac arrhythmia, mutations have been found in the genes encoding the potassium channels, human ether-a-go-go-related gene (HERG), and KVLQT1. The HERG protein is the pore-forming subunit of the cardiac rapidly activating delayed rectifier potassium channel. In both cases, mutations in the genes encoding these proteins result in a reduction of protein trafficking, and as a consequence a reduction in the amount of the relevant protein being integrated into the plasma membrane. As a result, cardiac cells expressing the mutant protein show reduced amplitude and altered voltage dependence of activation (Zhou *et al, J. Biol. Chem., 274(44)*, 31123-31126, 1999).

Mutations in various other membrane transport proteins have been suggested to cause a variety of other disorders due to mislocalization of the mutant protein, for example, glucose-galactose malabsorption, changes in cholesterol homeostasis, and defects in the multidrug transporter P-glycoprotein.

As membrane transport proteins are involved in essential cellular processes, and mutations affecting the function and/or localization of these proteins are involved in the aetiology of certain human diseases, there is a clear need in the art for methods of detecting mutations in these proteins and/or modulatory agents that affect their subcellular localization and turnover/recycling.

Known methods of determining the activity of a membrane transport protein generally involve the mere measurement of the movement of a specific substrate across a lipid bilayer, such as that found at the membrane of a cell. These methods are imprecise, as any redundancy in the transport process of interest, ie if a cell expresses multiple proteins that transport the same molecule, are not detected. For example, there are at least 12 hexose transporters encoded by the genes in the human genome and most mammalian cell types express more than one member of this family.

Alternatively, plasma membranes are usually isolated and low density microsomal fractions prepared. The membrane transport proteins are then photolabelled (eg bis-mannose photolabelling of GLUT4 located on the cell surface), and subsequently immunoprecipitated.

Alternatively, plasma membrane sheets are prepared for use in microscopic analysis (Cushman and Wardzala., J Biol Chem. 255:4758-4762 (1980); Holman, et al., J Biol Chem. 265:18172-18179 (1990); Robinson, et al., J Cell Biol. 117:1181-1196 (1992)).

These assays are both laborious and subject to inter-assay variability, and furthermore, are only semi-quantitative. Accordingly, the quantitative nature of these assays is limited. Furthermore, these assays are not readily adapted to high-throughput analysis, for example, for screening compounds that modulate translocation of a membrane transport protein.

Accordingly, there is a clear requirement in the art for a straightforward, reproducible method for the detection and estimation of the amount of a membrane transport protein translocated to the plasma membrane, or of membrane protein turnover. Preferred assays will not require sub-cellular fractionation or multiple labelling. Preferred assays will also be useful for determining mutations and/or agents that affect translocation of the membrane transport protein.

Summary of the Invention

In work leading up to the present invention, the inventors sought to develop an assay that detects the amount of a membrane transport protein incorporated into the plasma membrane of a cell compared to the total amount of that membrane transport protein within the cell, to thereby determine the amount of trafficking and/or turnover of the membrane transport protein at the plasma membrane.

As exemplified herein, the present inventors have developed an assay that is capable of determining the amount of any membrane transport protein, in particular the GLUT4 protein present at the plasma membrane of a cell relative to the total amount of the membrane transport protein within the cell. In one particular example of the inventive assay, the GLUT4 protein was labelled with a detectable tag (SEQ ID NO: 3) and transduced into mammalian cells using a retroviral system. Following transduction, a moderate amount of the recombinant GLUT4 protein was expressed, i.e. the GLUT4 protein was expressed at such a level so as not to hinder normal trafficking processes in the cell. In this exemplary assay format, 3T3-L1 adipocyte cells were used in the analysis of GLUT4 translocation as these cells are highly insulin responsive, and the response of these cells has been widely studied in the art. The amount of the tag in intact cells (i.e. cells that have not been permeabilized, or disrupted or lysed) compared to the amount of the tag in cells following their permeation was then determined. In this respect, the amount of tag present in the intact cell is indicative of the amount of membrane protein localized at the plasma membrane, whereas the amount of the tag present in the permeabilized cell is indicative of the total amount of membrane protein expressed in the cell.

In an exemplified embodiment, the GLUT4 protein was labelled with a tag consisting of a haemagglutinin (HA) peptide, by producing a fusion protein between GLUT4 and a peptide capable of binding an antibody that does not permeate the cell membrane. The HA tag was fused to an extracellular domain of the GLUT4 protein. In intact cells, the amount of HA tag detected by antibody binding represents the amount of membrane bound GLUT4, because the antibody cannot enter the cell. Following detection of membrane bound GLUT4, the cell membrane/s were disrupted or permealized, incubated with the antibody and the total amount of the tag antibody bound to the HA was then detected. This second reading reprsented the total GLUT4 in the cell (i.e. membrane-bound and intracellular GLUT4).

Alternatively, the total amount of membrane protein is determined by performing parallel reactions wherein the cells of one sample are permeabilized and contacted with the antibody that binds to the tag localized intracellularly and at the plasma membrane. A second sample is contacted with the tag in the absence of permeation of the cell to provide a measure of the amount of membrane-localised transport protein.

Accordingly, this assay facilitates the estimation of the relative amount of GLUT4 (or other membrane transport protein) that has been incorporated into the cell membrane.

Advantageously, the present assay is performed using a fluorescent detection device that enables rapid and reproducible detection of the tagged GLUT4. Accordingly, this system is readily adapted to high-throughput analysis of the translocation of a protein and agents that affect this process, for example, by using cells grown in 96-well or 384-well tissue culture plates.

As this system detects the amount of a protein that has been translocated to the plasma membrane of a cell relative to the total amount of that protein in a cell, results attained from an assay are readily compared, enabling rapid analysis of these results.

As will be apparent to the skilled artisan, while the assay described hereinbefore was developed specifically to analyze the translocation of GLUT4 in 3T3-L1 cells, it is equally applicable to the analysis of any membrane protein in any cell type.

Accordingly, one aspect of the present invention provides a method of determining the amount of a membrane transport protein translocated to the plasma membrane of a cell, said method comprising:

(a) determining the amount of a membrane transport protein at the plasma membrane by:
  (i) contacting the membrane transport protein with a ligand that binds to an extracellular domain of the membrane transport protein for a time and under conditions sufficient for the ligand to bind the membrane transport protein; and
  (ii) detecting the amount of ligand bound to the membrane transport protein;

(b) (i) permeabilizing or disrupting the plasma membrane of the cell and contacting the membrane transport protein within the cell with a ligand that binds to an extracellular domain of the membrane transport protein for a time and under

conditions sufficient for the ligand to bind the membrane transport protein; and

(ii) detecting the amount of ligand bound to the membrane transport protein within the cell; and

(c) comparing the amount of ligand detected at (a) (ii) and (b) (ii) to determine the amount of the membrane transport protein at the plasma membrane relative to the amount of the membrane transport protein inside the cell.

In one embodiment, the method of the present invention determines the amount of a labelled membrane transport protein translocated to the plasma membrane of a cell, said method comprising:

(a) determining the amount of the labelled membrane transport protein at the plasma membrane of a cell by:

(i) contacting the labelled membrane transport protein with a ligand of the label for a time and under conditions sufficient for the ligand to bind the label;

(ii) detecting the amount of ligand bound to the labelled membrane transport protein;

(b) (i) permeabilizing or disrupting the plasma membrane of the cell and contacting the labelled membrane transport protein within the cell with a ligand of the label for a time and under conditions sufficient for the ligand to bind the label;

(ii) detecting the amount of ligand bound to the labelled membrane transport protein within the cell; and

(c) comparing the amount of ligand detected at (a) (ii) and (b) (ii) to determine the amount of the labelled membrane transport protein at the plasma membrane relative to the amount of the labelled membrane transport protein inside the cell.

In an alternative embodiment of the present invention, the method is performed in parallel cellular samples. As will be apparent to the skilled artisan, when the amount of a protein at the plasma membrane of a cell and the amount of the protein in a cell are detected in different cell samples it is preferable that the assays are performed in the same cell type and the cells have been cultured in parallel. As used herein, the term "cultured in parallel" shall be taken to mean that the cells are cultured under the same or similar conditions, with regard to, for example, temperature, culture medium and any additional agents added to the culture medium.

This embodiment of the invention provides a method of determining the amount of a membrane transport protein translocated to the plasma membrane of a cell, said method comprising:

(a) determining the amount of the membrane transport protein at the plasma membrane of a cell by:
   (i) contacting a cell with a ligand that binds to an extracellular domain of the membrane protein for a time and under conditions sufficient for the ligand to bind the membrane transport protein; and
   (ii) detecting the amount of ligand bound to the membrane transport protein;
(b) determining the total amount of the membrane transport protein in another cell by:
   (i) permeabilizing or disrupting the other cell;
   (ii) contacting the membrane transport protein in the other cell with a ligand that binds to an extracellular domain of the membrane transport protein for a time and under conditions sufficient for the ligand to bind the membrane transport protein;
   (iii) detecting the amount of ligand bound to the membrane transport protein; and
(c) comparing the amount of ligand detected at (a) (ii) and (b) (iii) to determine the amount of the labelled membrane transport protein at the plasma membrane relative to the total amount of labelled membrane transport protein.

Accordingly, the amount of membrane transport protein detected at the plasma membrane is determined relative to the amount of membrane transport protein detected in the cell, for example as a percentage of the total amount of the membrane transport protein in the cell.

An alternative embodiment of the invention provides a method of determining the amount of a labelled membrane transport protein translocated to the plasma membrane of a cell, said method comprising:

(a) determining the amount of a labelled membrane transport protein at the plasma membrane of a cell by:
   (i) contacting a portion of the cell with a ligand of the label for a time and under conditions sufficient for the ligand to bind the label; and
   (ii) detecting the amount of ligand bound to the labelled membrane transport protein;

(b) determining the total amount of the labelled membrane transport protein in another cell by:

(i) permeabilizing or disrupting the other cell;

(ii) contacting the labelled membrane transport protein in the other cell with a ligand of the label for a time and under conditions sufficient for the ligand to bind the label;

(iii) detecting the amount of ligand bound to the labelled membrane transport protein in the other cell; and

(c) comparing the amount of ligand detected at (a) (ii) and (b) (iii) to determine the amount of the labelled protein at the plasma membrane relative to the total amount of labelled protein.

As will be apparent to a person skilled in the art, it is preferable that this assay is performed *in vitro*.

As used herein the term "membrane transport protein" shall be taken to mean a peptide, polypeptide or protein that catalyzes the movement of a molecule across a membrane, whether this movement is by diffusion (simple or facilitated) or active transport. Membrane transport proteins in the present context exist as intracellular proteins and are capable of being membrane-localized. Such a protein may be, for example, a channel, a transporter, an ATP pump, a symporter or an antiporter.

Preferably, the method described *supra* further comprises the first step of providing a cell that is capable of expressing the membrane transport protein in a format suitable for labelling, or as a tagged or labelled protein, or otherwise capable of binding to a ligand via a tag or extracellular domain.

As used herein, the term "providing a cell" shall be understood to mean introducing a nucleic acid into a cell that encodes the protein fused to a label, or alternatively introducing the protein linked to a label into a cell, or expressing the membrane transport protein or a tagged or labelled form thereof.

In a preferred embodiment, the amount of a labelled membrane transport protein detected at the plasma membrane of a cell is detected without disrupting the plasma membrane. Accordingly, it is preferable that the label associated with that protein is

situated such that it is detectable extracellularly (for example, a GLUT4 protein may have a label associated with the first exofacial loop).

It is particularly preferred that the membrane transport protein is expressed as a fusion protein with the label. Furthermore, it is particularly preferred that only one type of label is associated with a membrane transport protein. As used herein, the term "one type of label" shall be taken to mean that only one form of label, eg a c-myc tag or a HA tag, is associated with a particular membrane transport protein in the inventive assay, however multiple distinct labels can be used in multiplex assays of different membrane transport proteins, or alternatively to measure the amount of translocation of one or more membrane transport proteins relative to a negative control protein. Several copies of this "one type of label" may also be associated with the membrane transport protein, (e.g. 6 consecutive HA tags).

It is to be understood that the ligand of either the membrane protein or the label should be selected such that it is unable to enter the cell prior to the membrane being permeabilized. As used herein the term "ligand" shall be taken in its broadest context to include any chemical compound, polynucleotide, peptide, protein, lipid, carbohydrate, small molecule, natural product, polymer, etc. that is able to bind selectively and stoichiometrically, whether covalently or not, to the label. The ligand may bind to its target via any means including hydrophobic interactions, hydrogen bonding, electrostatic interactions, van der Waals interactions, pi stacking, covalent bonding, or magnetic interactions amongst others.

As will be apparent to the skilled artisan, the present method also provides means to monitor or determine the turnover (or recycling) of a membrane transport protein in a plasma membrane. As used herein, the terms "turnover of a membrane transport protein in a plasma membrane" or recycling of a membrane transport protein in a plasma membrane shall be taken to mean that the method may be used to determine the rate at which a membrane transport protein is translocated to a membrane and then subsequently translocated from the plasma membrane to another site in the cell.

Accordingly, a further embodiment of the present invention provides a method of determining the amount of a membrane transport protein recycled from the plasma membrane of a cell. This method comprises detecting the amount of a membrane transport protein that has translocated to the plasma membrane in a cell at various

points in time. By comparing the amount of a membrane transport protein that has translocated to the plasma membrane at these time points, the rate of translocation to the membrane and recycling from the membrane is determined.

In one embodiment, this method is useful for determining the amount of a labelled membrane transport protein at the plasma membrane prior to and after treatment of a cell (or cells) with an agent, or incubation of a cell (or cells) under a condition that affects protein translocation.

A further embodiment of the present invention provides a method of determining a mutation in a nucleic acid encoding a membrane transport protein wherein the mutation modulates the amount of the membrane transport protein translocated to the plasma membrane of a cell. This method comprises detecting the amount of a membrane transport protein that has translocated to the plasma membrane in a cell that expresses a tagged and/or labelled mutant form of the membrane transport protein to the amount of the membrane transport protein translocated to the plasmsa membrane in a cell expressing a tagged and/or labelled wild-type membrane transport protein. By comparing the amount of a membrane protein that has translocated to the plasma membrane in each of these cells, the effect of said mutation on translocation of the membrane transport protein to the plasma membrane is determined.

As used herein, the term "wild type" membrane transport protein" shall be taken to mean a membrane transport protein that is an unmutated form of the mutated membrane transport protein that is being assayed.

A related embodiment of the invention provides a method of determining a cell that comprises a mutation in a gene that modulates the amount of a membrane transport protein translocated to the plasma membrane of the cell. This method comprises performing the method of the invention to determine the amount of a tagged and/or labelled membrane transport protein that translocates to the membrane in a test sample, and the amount of a tagged and/or labelled membrane transport protein that translocates to the membrane in a control sample. Wherein the test sample comprises more, or less, tagged and/or labelled membrane transport protein at the plasma membrane relative to the control sample, the cell will comprise a mutation in a gene that affects translocation of the membrane transport protein to the plasma membrane.

As used herein the term "test cell" shall be understood to mean a cell that is being tested or analysed to determine whether or not it shows increased or decreased translocation of the membrane transport protein compared to a control cell. Such a cell may be derived from a subject that is suffering from a disorder that is thought to be caused by a mutation that is caused by a mutation that affects translocation of a membrane transport protein.

As used herein, the term "control cell" shall be taken to mean a cell that is the same cell type or is isogenic with the test cell, and does not have a cellular defect affecting the activity or translocation of the membrane transport protein being assayed. In one embodiment, the control cell is not infected with the retrovirus, and as a consequence does not express the recombinant membrane transport protein of interest. Alternatively, a control cell may be the same cell as that transformed with a nucleic acid encoding the membrane transport protein of interest, however is additionally transformed with a nucleic acid encoding a control membrane transport protein other than the protein of interest. Preferably, the second membrane transport protein does not translocate to the membrane under the same conditions as the membrane transport protein of interest. For example, in studying GLUT4 translocation, a second membrane transport protein may be a transferrin receptor that is assayed to provide a baseline activity against which GLUT4 translocation is determined.

When the method of the present invention is used to determine an agent that modulates the translocation of a membrane transport protein, the skilled artisan will be aware that a control cell may be a cell that is not exposed to said agent, or alternatively, a cell that is exposed to an agent that is known to modulate the translocation of the membrane transport protein.

As different cell types may behave differently with respect to trafficking, the present invention clearly encompasses performing the inventive method in different cell types.

A further aspect of the present invention provides a method of determining an agent that modulates the amount of a protein translocated to the plasma membrane of a cell. This method comprises the use of any of the previously described methods to determine the amount of a tagged and/or labelled membrane transport protein translocated to the plasma membrane in a cell that is exposed to an agent relative to the amount of tagged and/or labelled membrane transport protein translocated in a cell that is not exposed to

the agent. An agent that causes more or less membrane transport protein to be translocated to the plasma membrane than observed in a control cell is a modulator of the translocation of said membrane transport protein.

Accordingly, this aspect of the present invention provides a method of determining an agent that modulates a method of determining an agent that modulates translocation of a membrane transport protein to the plasma membrane of a cell said method comprising:

(a) performing a method of the present invention to determine translocation of a wild-type or mutant form of the membrane transport protein in the presence of a candidate agent; and

(b) comparing translocation of the membrane transport protein at (a) to translocation of the wild-type or mutant membrane transport protein in the absence of the candidate agent, wherein a difference in translocation indicates that the candidate agent modulates translocation of the membrane transport protein.

In a preferred embodiment, this aspect of the present invention provides a method of determining an agent that modulates translocation of a membrane transport protein to the plasma membrane of a cell said method comprising:

(a) performing a method of the present invention to determine translocation of a wild-type or mutant form of the membrane transport protein in the absence of a candidate agent;

(b) performing a method of the present invention to determine translocation of a wild-type or mutant form of the membrane transport protein in the presence of the candidate agent; and

(c) comparing translocation of the membrane transport protein at (a) and (b) wherein a difference in translocation indicates that the candidate agent modulates translocation of the membrane transport protein.

Accordingly, such a method is of particular use in determining a potential therapeutic agent that is capable of modulating the translocation of a membrane transport protein to the plasma membrane. A method of determining such an agent may be of particular relevance in developing therapeutics for diseases such as, for example, type II diabetes mellitus, cystic fibrosis, and a nephrotic syndrome characterized by absence of aquaporin II protein from the apical membrane.

A still further aspect of the present invention provides a kit for determining an agent that modulates the amount of a protein that is translocated to the plasma membrane of a cell.

The present invention clearly encompasses the use of a gene construct that encodes a tagged membrane transport protein in the inventive method described herein. Such gene constructs may have been produced for the other applications, or be new. This invention clearly extends to any novel gene constructs described herein without limitation to their use.

Furthermore, the present invention clearly encompasses the use of a cell that expresses a tagged membrane transport protein in the inventive method. Such cells may have been produced for the other applications, or be new. This invention clearly extends to any novel cells described herein without limitation to their use.

Brief description of the figures

Figure 1A is a schematic representation of a recombinant GLUT4 protein that is labelled with a HA epitope. Note that when expressed in a cell the HA epitope is within an extracellular domain of the protein. This location of the HA epitope facilitates detection of the GLUT4 protein when translocated to the plasma membrane without disrupting said plasma membrane.

Figure 1B is a schematic representation showing the various forms of GLUT4 used in the analysis of translocation of GLUT4 to the plasma membrane. WT represents the wild-type form of GLUT4 (SEQ ID NO: 1) TAIL represents a mutant form of GLUT4 in which the residues at the C-terminus of GLUT4 have been mutated (SEQ ID NO: 5); L489,490A represents a mutant form of GLUT4 in which a di-leucine motif at the C-terminal end of GLUT4 has been mutated to a di-Alanine motif (SEQ ID NO: 6); and F5A represents a mutant form of GLUT4 in which the phenylalanine at amino acid number 5 of GLUT4 has been mutated to Alanine (SEQ ID NO: 7), wherein each of these proteins have been labelled with a HA epitope tag (SEQ ID NO: 18) in an intracellular domain, for example, the sequence of a WT, GLUT4 labelled with an HA epitope tag is represented by SEQ ID NO: 3.

Figure 2 is a schematic representation of the method of detecting the amount of GLUT4 that has translocated to the plasma membrane. The left hand side of the figure shows a

cell that is stained to determine the amount of GLUT4 that has translocated to the membrane. Recombinant GLUT4 labelled with a HA epitope is expressed in the cell; the cell is then fixed and the GLUT4 that has translocated to the plasma membrane is detected with an anti-HA antibody; the cell is then permeabilized with saponin and the anti-HA antibody detected with a fluorescent secondary antibody. The right hand side of the figure shows a cell that is used to determine the total amount of GLUT4 in a cell. Recombinant GLUT4 labelled with a HA epitope is expressed in the cell; the cell is then fixed; and permeabilized with saponin. The HA epitope is then detected with an anti-HA antibody, which is now able to enter the cell. The anti-HA epitope is then detected with a fluorescent secondary antibody. Comparing the results obtained from the two cells shows the amount of GLUT4 that has translocated to the plasma membrane as a function of total GLUT4.

Figure 3 is a copy of a photographic representation of various cells used to analyze the translocation of GLUT4. The top row of cells are 3T3-L1 fibroblasts and the bottom row 3T3-L1 adipocytes. From left to right the cells were not transduced (i.e. do not express a tagged GLUT4); were transduced with a tagged WT, GLUT4; were transduced with a tagged TAIL mutant GLUT4; were transduced with a tagged L489,490A mutant GLUT4; or were transduced with a tagged F5A mutant GLUT4.

Figure 4A is a graphical representation of the effect of insulin that do not express HA-tagged GLUT4. The amount of fluorescence detected using the anti-HA antibody (HA) was the same as that detected with a non-relevant (NR) antibody, indicating that the anti-HA antibody does not non-specifically bind a protein in the cell.

Figure 4B is a graphical representation of the amount of HA tagged GLUT4 detected at the plasma membrane of 3T3-L1 adipocytes incubated in the presence of 200 nM insulin. Over time, the amount of HA-tagged GLUT4 (squares) detected at the plasma membrane increased, while the amount of the non-relevant protein (triangles) remained constant. This indicates that insulin induces GLUT4 translocation to the plasma membrane.

Figure 4C is a graphical representation of the percentage of total GLUT4 in a cell that has translocated the plasma membrane in the presence of 200 nM insulin. Using the method described herein the amount of HA tagged GLUT4 that was translocated to the

plasma membrane in the presence of insulin was determined relative to the total HA-tagged GLUT4 in a cell.

Figure 4D is a graphical representation of the percentage of total GLUT4 in a cell that has translocated to the plasma membrane in the presence of various concentrations of insulin. Using the method described herein the effect of insulin concentration on the amount of HA-tagged GLUT4 translocation to the plasma membrane relative to the total HA-tagged GLUT4 was determined (triangle). In the presence of wortmannin (squares) insulin induced translocation of GLUT4 was almost totally abrogated.

Figure 5A is a graphical representation showing the amount of a HA-tagged form of GLUT4 (from left to right: WT; TAIL; L489; 490A; and F5A) was detected at the plasma membrane of 3T3-L1 fibroblasts at relative to the total HA-tagged form of GLUT4. Clearly GLUT4 translocation is induced by insulin in fibroblasts.

Figure 5B is a graphical representation showing the percentage of a HA-tagged form of GLUT4 (from left to right: WT; TAIL; L489; 490A; and F5A) at the plasma membrane of 3T3-L1 adipocytes in the presence of 200 nM insulin. Interestingly, the L489; L490A and F5A mutants, which are believed to be impaired in their internalisation/recycling, show an increase in adipocytes compared with fibroblasts (Figure 4A).

Detailed description of the preferred embodiments

One aspect of the present invention provides a method of determining the amount of a membrane transport protein translocated to the plasma membrane of a cell, said method comprising:

(a) determining the amount of a membrane transport protein at the plasma membrane by:
   (i) contacting the membrane transport protein with a ligand that binds to an extracellular domain of the membrane transport protein for a time and under conditions sufficient for the ligand to bind the membrane transport protein; and
   (ii) detecting the amount of ligand bound to the membrane transport protein;

(b) (i) permeabilizing or disrupting the plasma membrane of the cell and contacting the membrane transport protein within the cell with a ligand that binds to an extracellular domain of the membrane transport protein for a time

and under conditions sufficient for the ligand to bind the membrane transport protein; and

(ii) detecting the amount of ligand bound to the membrane transport protein within the cell; and

(c) comparing the amount of ligand detected at (a) (ii) and (b) (ii) to determine the amount of the membrane transport protein at the plasma membrane relative to the amount of the membrane transport protein inside the cell.

Alternatively, this method may be performed in cells that have been cultured in parallel. Accordingly, one embodiment of the invention provides a method of determining the amount of a membrane transport protein translocated to the plasma membrane of a cell, said method comprising:

(a) determining the amount of the membrane transport protein at the plasma membrane of a cell by:

(i) contacting a cell with a ligand that binds to an extracellular domain of the membrane protein for a time and under conditions sufficient for the ligand to bind the membrane transport protein; and

(ii) detecting the amount of ligand bound to the membrane transport protein;

(b) determining the total amount of membrane transport protein by:

(i) permeabilizing or disrupting another cell;

(ii) contacting the membrane transport protein with a ligand that binds to an extracellular domain of the membrane transport protein for a time and under conditions sufficient for the ligand to bind the membrane transport protein;

(iii) detecting the amount of ligand bound to the membrane transport protein; and

(c) comparing the amount of ligand detected at (a) (ii) and (b) (iii) to determine the amount of the labelled membrane transport protein at the plasma membrane relative to the total amount of labelled membrane transport protein.

In accordance with this embodiment, it is preferable that the ligand of the membrane transport protein binds to an extracellular domain of the membrane transport protein, such as, for example, the monoclonal antibodies mAb5 and mAb263 that specifically bind an extracellular region of the growth hormone receptor protein (available from AGEN Limited, Acacia Ridge, Queensland, Australia). Furthermore, it is additionally preferred that the ligand is unable to enter an intact cell. Accordingly, when used to detect a membrane transport protein of interest in an intact cell, such a ligand only binds to the membrane transport protein that has translocated to the plasma membrane.

Following permeabilization or disruption of the cell membrane such a ligand is capable of entering the cell and detecting the total amount of that membrane transport protein.

In one embodiment, a ligand is an antibody that specifically binds to the ligand. As used herein the term "antibody" refers to intact monoclonal or polyclonal antibodies, immunoglobulin (IgA, IgD, IgG, IgM, IgE) fractions, humanized antibodies, or recombinant single chain antibodies, as well as fragments thereof, such as, for example Fab, F(ab)2, and Fv fragments. For example, an epitope tag may be detected using an antibody that specifically binds to that tag. Anti-FLAG and anti-c-myc monoclonal antibodies are available from Sigma-Aldrich (Sydney, Autralia); an anti-HA tag is available from Zymed (South San Francisco, CA 94080); and an anti-V5 monoclonal antibody is available from Serotec (Oxford, UK). These antibodies may be labelled using a suitable reporter molecule, such as, for example, an enzyme (such as horseradish peroxidase or alkaline phosphatase), a substrate, a cofactor, an inhibitor, a dye, a radionucleotide, a luminescent group, a fluorescent group, biotin or a colloidal particle, such as colloidal gold or selenium. Alternatively, these antibodies may be detected using a secondary or even tertiary antibody that has been labelled with a suitable reporter molecule. Methods of detecting the reporter molecule are well known to one skilled in the art, and are described, for example, in Scopes (*In:* Protein purification: principles and practice, Third Edition, Springer Verlag, 1994).

In another embodiment, the ligand is a peptide, polypeptide or protein that is capable of binding a label. For example, biotin is capable of binding strepavidin and strepavidin is capable of binding the peptide represented by SEQ ID NO: 23. Accordingly, the membrane transport protein of interest is fused to such a label and subsequently detected with a ligand of the label. These ligands may be labelled using a suitable reporter molecule, such as, for example, an enzyme (such as horseradish peroxidase or alkaline phosphatase), a substrate, a cofactor, an inhibitor, a dye, a radionucleotide, a luminescent group, a fluorescent group, biotin or a colloidal particle, such as colloidal gold or selenium. Methods of detecting the reporter molecule are well known to one skilled in the art, and are described, for example, in Scopes (*In:* Protein purification: principles and practice, Third Edition, Springer Verlag, 1994).

In a further embodiment, a ligand is a substrate for an enzymatic reaction. For example the label may be the protein β-galactosidase. Detection of the amount of membrane transport protein either at the plasma membrane or the total amount of a labelled

membrane transport protein is facilitated using 5-bromo-4-chloro-3-indol-beta-D-galaotopyranoside (x-gal). Other labels of use in such an assay include, horseradish peroxidase and alkaline phosphatase.

It is to be understood that the ligand is not capable of entering the cell in which the assay is performed without the membrane of said cell being permeabilized or disrupted. Accordingly, prior to permeabilization/disruption of the cell membrane the ligand of the label will only bind the label that is external to the cell (representing a membrane transport protein that has been translocated to the plasma membrane).

Clearly, this invention relates to an invention that determines the amount of a membrane transport protein that has translocated to the plasma membrane, compared to the total amount of that membrane transport protein in the cell (including the amount that has translocated to the plasma membrane).

In a preferred embodiment, the membrane transport protein is a protein that translocates to the plasma membrane under normal physiological conditions, or following stimulation by a condition or agent, such as, for example, glucose or insulin. Preferably the membrane transport protein is, for example, an ABC transporter protein, a P class ATP pump, a F class ATP pump, a V class ATP pump, a Cl^- channel, a H^+ channel and Ca^{++} channel, a K^+ channel, an uniporter a symporter or an antiporter. For example, the membrane transport protein is a membrane transport protein selected from the group consisting of ABC1, ABCA2, ABCA3, ABCR, ABCA5, ABCA6, ABCA7, ABCA8, ABCA9, ABCA10, ABCA12, ABCA13, PGY1, TAP1, TAP2, PGY3, ABCB5, ABCB6, ABC7, M-ABC1, ABCB9, ABCB10, BSEP, MRP1, MRP2, MRP3, MRP4, MRP5, MRP6, CFTR, SUR1, SUR2, ABCC10, ABCC11, ABCC12, ABCC13, ALD, ALDL1, ABCD2, PXMP1, PXMP1L, RNASELI, ABC50, ABCF2, ABCF3, ABCG1, ABCG2, ABCG4, ABCG5, ABCG8, KCNA1, CACNL1A4, CACNL1A4, CACNL1A4, KCNQ2, KCNQ3, SCN1B, CHRNA4, GLRA1, KCNE1, KCNQ4, SCN4A, SCN4A, SCN4A, CACNL1A3, CLCN1, CNCN1, RYR1, RYR1, CHRNA1, KCNQ1, HERG, SCN5A, KCNE1, SCN5A, KCNE1, GLUT1, GLUT2, GLUT3, GLUT4, GLUT5, GLUT6, GLUT7, GLUT8, GLUT9, GLUT10, GLUT11, GLUT12, HMIT.

As used herein, the nomenclature for GLUT proteins and HMIT is described by Joost *et al, 2001, Am. J. Physiol. Endocrinol. Metab. 282*: E974-E976, 2002.

In a particularly preferred embodiment, the membrane transport protein is GLUT4 (SEQ ID NO: 1) or a protein encoded by the nucleic acid represented by SEQ ID NO: 2.

In one embodiment, the membrane transport protein is native to the cell. As used herein, the term "native to the cell" shall be taken to mean that the membrane transport protein is an endogenous membrane transport protein of the cell, or that it is normally expressed by the cell.

In another embodiment, the membrane transport protein is a recombinant membrane transport protein. In accordance with this embodiment, the membrane transport protein may be directly introduced into the cell using methods well known in the art and/or described herein. Alternatively, the membrane transport protein may be expressed within the cell using a gene construct as described herein, using methods known in the art and/or described herein.

In a preferred embodiment, the method of the present invention determines the amount of a labelled membrane transport protein translocated to the plasma membrane of a cell, said said method comprising:

(a) providing a cell comprising a labelled membrane transport protein;

(b) determining the amount of the labelled membrane transport protein at the plasma membrane by:

 (i) contacting the labelled membrane transport protein with a ligand of the label for a time and under conditions sufficient for the ligand to bind the label;

 (ii) detecting the amount of ligand bound to the labelled membrane transport protein;

(c) (i) permeabilizing or disrupting the plasma membrane of the cell and contacting the labelled membrane transport protein within the cell with a ligand of the label for a time and under conditions sufficient for the ligand to bind the label;

 (ii) detecting the amount of ligand bound to the labelled membrane transport protein within the cell; and

(d) comparing the amount of ligand detected at (b) (ii) and (c) (ii) to determine the amount of the labelled membrane transport protein at the plasma membrane relative to the amount of the labelled membrane transport protein inside the cell.

In an alternative embodiment of the present invention, the method is performed in parallel cellular samples.

This embodiment of the invention provides a method of determining the amount of a labelled protein translocated to the plasma membrane of a cell, said method comprising:

(a) providing cells comprising a labelled membrane transport protein;

(b) determining the amount of the labelled membrane transport protein at the plasma membrane by:

    (i) contacting a portion of the cells at (a) with a ligand of the label for a time and under conditions sufficient for the ligand to bind the label; and

    (ii) detecting the amount of ligand bound to the labelled membrane transport protein;

(c) determining the total amount of labelled membrane transport protein by:

    (i) permeabilizing or disrupting the plasma membrane of a portion of the cells at (a);

    (ii) contacting the labelled membrane transport protein with a ligand of the label for a time and under conditions sufficient for the ligand to bind the label;

    (iii) detecting the amount of ligand bound to the labelled membrane transport protein; and

(d) comparing the amount of ligand detected at (b) (ii) and (c) (iii) to determine the amount of the labelled membrane transport protein at the plasma membrane relative to the total amount of labelled membrane transport protein.

In another preferred embodiment, the membrane transport protein is a mutant form of a membrane transport protein that occurs in nature.

In one embodiment, the membrane transport protein is a mutant form of a membrane transport protein that has a reduced rate of removal from the cell surface (internalization). As used herein, the term "reduced rate of transporter internalization" shall be taken to mean that the membrane transport protein has been mutated in such a way that following translocation to the membrane it is not internalized or endocytosed, i.e. translocated away from the membrane at the same rate as the wild-type form of the membrane transport protein, rather it is internalized at a slower rate. For example, mutant forms of GLUT4 that have a reduced rate of transporter internalization include the L489, 490A mutant (SEQ ID NO:6) and the F5A mutant (SEQ ID NO: 7). Such

mutants are of particular use in the methods of the present invention as they accumulate at the plasma membrane, effectively amplifying or increasing the amount of membrane transport protein detected. Accordingly, such mutants are of use in the detection of a minor change (i.e. increase or decrease) of the translocation of a membrane transport protein, for example, when screening for a modulator of translocation of a membrane transport protein.

In the case of GLUT4, wild-type GLUT4 is more effectively translocated and recycled in the presence of insulin, as would be expected. Accordingly, wild-type GLUT4 is more effective in an assay for determining changes in translocation in the presence and/or absence of insulin, for example, when screening for a compound/agent that modulates GLUT4 translocation in the presence of insulin.

In one embodiment, the membrane transport protein is a membrane transport protein that is rapidly translocated and recycled, whether that membrane transport protein is a wild-type or mutant form.

In one embodiment of the invention, it is preferable that the membrane transport protein is labelled to facilitate detection of said membrane transport protein.

As used herein, the term "labelled membrane transport protein" shall be taken to mean a membrane transport protein that is covalently linked with a moiety that is detectable. Examples of such moieties include a tag selected from the group consisting of influenza virus hemagglutinin (HA) (YPYDVPDYA, SEQ ID NO: 8), Simian Virus 5 (V5) (GKPIPNPLLGLDST, SEQ ID NO: 9), polyhistidine (eg 6xHis), c-myc (FQKLISEEDL, SEQ ID NO: 10), FLAG (DYKDDDDKC, SEQ ID NO: 11), the epitope tags described by Slootstra *et al. Mol Divers 2(3)*:156-164, 1997, MDFKDDDDK (SEQ ID NO: 12) and MDYKAFDNL (SEQ ID NO: 13), GST (glutathione-S-transferase), (SEQ ID NO: 14), MBP (maltose binding protein) (SEQ ID NO: 15), GAL4 (SEQ ID NO: 16), β-galactosidase (SEQ ID NO: 17), enhanced green fluorescence protein (eGFP) (SEQ ID NO: 18), yellow fluorescent protein (SEQ ID NO: 19), soluble modified blue fluorescent protein (SEQ ID NO: 20), soluble-modified red-shifted green fluorescent protein (SEQ ID NO: 21), and cyan fluorescent protein (SEQ ID NO: 22). Alternatively, the membrane transport protein may be labelled with a protein that directly associates with another known protein, such as for example,

biotin, strepavidin or the Strep-Tag, an 8 amino acid strepavidin binding sequence (WSHPQFEK, SEQ ID NO: 23) (available from Sigma-Genosys, Sydney, Australia).

In a particularly preferred embodiment, the label covalently linked to a membrane transport protein is a HA tag (SEQ ID NO: 8).

In another embodiment, the method of the present invention determines the amount of a labelled membrane transport protein translocated to the plasma membrane of a cell, said method comprising:

(a) providing a cell comprising a labelled membrane transport protein;

(b) determining the amount of the labelled membrane transport protein at the plasma membrane by:

  (i) contacting the labelled membrane transport protein with a ligand of the label for a time and under conditions sufficient for the ligand to bind the label;

  (ii) detecting the amount of ligand bound to the labelled membrane transport protein;

(c) (i) permeabilizing or disrupting the plasma membrane of the cell and contacting the labelled membrane transport protein within the cell with a ligand of the label for a time and under conditions sufficient for the ligand to bind the label;

  (ii) detecting the amount of ligand bound to the labelled membrane transport protein within the cell; and

(d) comparing the amount of ligand detected at (b) (ii) and (c) (ii) to determine the amount of the labelled membrane transport protein at the plasma membrane relative to the amount of the labelled membrane transport protein inside the cell.

Alternatively, this process may be performed in cells cultured in parallel. Accordingly one embodiment of the invention provides a method of determining the amount of a labelled membrane transport protein translocated to the plasma membrane of a cell, said method comprising:

(a) providing cells comprising a labelled membrane transport protein;

(b) determining the amount of the labelled membrane transport protein at the plasma membrane by:

  (i) contacting a portion of the cells at (a) with a ligand of the label for a time and under conditions sufficient for the ligand to bind the label; and

  (ii) detecting the amount of ligand bound to the labelled membrane transport protein;

(c) determining the total amount of labelled membrane transport protein by:

(i) permeabilizing or disrupting a portion of the cells at (a);

(ii) contacting the labelled membrane transport protein with a ligand of the label for a time and under conditions sufficient for the ligand to bind the label;

(iii) detecting the amount of ligand bound to the labelled membrane transport protein; and

(d) comparing the amount of ligand detected at (b) (ii) and (c) (iii) to determine the amount of the labelled protein at the plasma membrane relative to the total amount of labelled protein.

In one embodiment a label is detected using a ligand of the label. As used herein the term "ligand of the label" shall be taken to mean a ligand that specifically binds the label covalently bound to the membrane transport protein of interest.

In order to detect the amount of a membrane transport protein that has translocated to the plasma membrane of a cell it is important that the tag is situated such that only the membrane transport protein that has translocated to the membrane is detected. Accordingly, it is preferable that the tag is situated on an extracellular region of the membrane transport protein of interest. In this respect, when initially detecting the label, as the plasma membrane of the cell is intact (or has not been disrupted), only the label that is extracellular will be detected.

Accordingly, it is necessary to determine a region of the membrane transport protein that is extracellular. Methods of determining the region of a membrane transport protein that is extracellular are known in the art and are described, for example, in Nakashima and Nishikawa, *FEBS Lett.* *303*: 141-146, 1992; Nakashima and Nishikawa, *J. Mol. Biol.*, *238*: 54-61, 1994 or Rost *et al*, *Prot Sci.*, *4*: 521-533, 1995. Such methods rely upon the analysis of the amino acid composition of a membrane transport protein to determine those regions that are extracellular and those regions that are intracellular.

In a particularly preferred embodiment, the membrane transport protein GLUT4 is labelled with a HA tag that is situated within the first extracellular domain (or first exofacial loop) as represented by SEQ ID NO:3.

In one embodiment, the label and the membrane transport protein are each produced as

separate moieties and covalently linked, using, for example disulphide linkage. As will be apparent to the person skilled in the art such a membrane transport protein must then be delivered to the cell. In one embodiment the peptide encoded by the nucleic acid fragment of the present invention is expressed as a fusion protein with a peptide sequence capable of enhancing, increasing or assisting penetration or uptake of the protein by cells. Means and methods of enhancing, increasing or assisting penetration or uptake of the membrane transport protein by cells are described, for example. In Morris *et al, Nature Biotechnology 19,* 1173-1176, 2001.

In a particularly preferred embodiment of the present invention, the membrane transport protein is expressed as a fusion protein with the label. Methods of producing a fusion protein are well known in the art and are described, for example, in Sambrook *et al* (In: Molecular Cloning: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, New York, Third Edition 2001) and described herein. Essentially, the method of producing a fusion protein involves the generation of a gene construct that encodes the membrane transport protein of interest and the label such that they are expressed as a single protein.

In another embodiment an unlabelled membrane transport protein is expressed as a recombinant membrane transport protein. Essentially the method of expressing a recombinant protein comprises introducing a gene construct encoding the protein of interest into the cell and expressing said protein.

The term "gene construct" is to be taken in its broadest context and includes a promoter sequence that is placed in operable connection with a nucleic acid fragment of the present invention.

The term "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences of a genomic gene, including the TATA box or initiator element, which is required for accurate transcription initiation, with or without additional regulatory elements (ie. upstream activating sequences, transcription factor binding sites, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue specific manner. In the present context, the term "promoter" is also used to describe a recombinant, synthetic or fusion molecule, or derivative which confers, activates or enhances the expression of a nucleic acid molecule to which it is operably linked, and which encodes the peptide or protein.

Preferred promoters can contain additional copies of one or more specific regulatory elements to further enhance expression and/or alter the spatial expression and/or temporal expression of said nucleic acid molecule.

Placing a nucleic acid molecule under the regulatory control of, ie., "in operable connection with", a promoter sequence means positioning said molecule such that expression is controlled by the promoter sequence. Promoters are generally positioned 5' (upstream) to the coding sequence that they control. To construct heterologous promoter/structural gene combinations, it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting, ie., the gene from which the promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting, ie., the gene from which it is derived. Again, as is known in the art, some variation in this distance can also occur.

Typical promoters suitable for expression in viruses of bacterial cells and bacterial cells such as for example a bacterial cell selected from the group comprising *E. coli, Staphylococcus sp, Corynebacterium sp., Salmonella sp., Bacillus sp.,* and *Pseudomonas sp.*, include, but are not limited to, the *lacz* promoter, the Ipp promoter, temperature-sensitive λ_L or λ_R promoters, T7 promoter, T3 promoter, SP6 promoter or semi-artificial promoters such as the IPTG-inducible *tac* promoter or lacUV5 promoter.

Typical promoters suitable for expression in yeast cells such as for example a yeast cell selected from the group comprising *Pichia pastoris, S. cerevisiae* and *S. pombe*, include, but are not limited to, the *ADH1* promoter, the *GAL1* promoter, the *GAL4* promoter, the *CUP1* promoter, the *PHO5* promoter, the *nmt* promoter, the *RPR1* promoter, or the *TEF1* promoter.

Typical promoters suitable for expression in insect cells, or in insects, include, but are not limited to, the OPEI2 promoter, the insect actin promoter isolated from *Bombyx muri*, the *Drosophila sp. dsh* promoter (Marsh *et al Hum. Mol. Genet. 9*, 13-25, 2000) and the inducible metallothionein promoter. Preferred insect cells for expression of the recombinant polypeptides include an insect cell selected from the group comprising,

BT1-TN-5B1-4 cells, and *Spodoptera frugiperda* cells (eg., sf19 cells, sf21 cells).

Promoters for expressing peptides in plant cells are known in the art, and include, but are not limited to, the *Hordeum vulgare* amylase gene promoter, the cauliflower mosaic virus 35S promoter, the nopaline synthase (NOS) gene promoter, and the auxin inducible plant promoters P1 and P2.

Typical promoters suitable for expression in a virus of a mammalian cell, or in a mammalian cell, mammalian tissue or intact mammal include, for example a promoter selected from the group consisting of, retroviral LTR elements, the SV40 early promoter, the SV40 late promoter, the cytomegalovirus (CMV) promoter, the CMV IE (cytomegalovirus immediate early) promoter, the $EF_{1\alpha}$ promoter (from human elongation factor 1α), the EM7 promoter, the UbC promoter (from human ubiquitin C).

Preferred mammalian cells for expression of the nucleic acid fragments include epithelial cells, fibroblasts, kidney cells, T cells, or erythroid cells, including a cell line selected from the group consisting of COS, CHO, murine 10T, MEF, NIH3T3, MDA-MB-231, MDCK, HeLa, K562, HEK 293, 3T3-L1 and 293T. The use of neoplastic cells, such as, for example, leukemic/leukemia cells, is also contemplated herein.

Numerous expression vectors for expression of recombinant polypeptides in bacterial cells and efficient ribosome binding sites have been described, such as for example, PKC30 (Shimatake and Rosenberg, *Nature 292*, 128, 1981); pKK173-3 (Amann and Brosius, *Gene 40*, 183, 1985), pET-3 (Studier and Moffat, *J. Mol. Biol. 189*, 113, 1986); the pCR vector suite (Invitrogen), pGEM-T Easy vectors (Promega), the pL expression vector suite (Invitrogen) the pBAD/TOPO; the pFLEX series of expression vectors (Pfizer nc., CT,USA); the pQE series of expression vectors (QIAGEN, CA, USA), or the pL series of expression vectors (Invitrogen), amongst others.

Expression vectors for expression in yeast cells are preferred and include, but are not limited to, the pACT vector (Clontech), the pDBleu-X vector, the pPIC vector suite (Invitrogen), the pGAPZ vector suite (Invitrogen), the pHYB vector (Invitrogen), the pYD1 vector (Invitrogen), and the pNMT1, pNMT41, pNMT81 TOPO vectors (Invitrogen), the pPC86-Y vector (Invitrogen), the pRH series of vectors (Invitrogen), pYESTrp series of vectors (Invitrogen). A number of other gene construct systems for expressing the nucleic acid fragment of the invention in yeast cells are well-known in

the art and are described for example, in Giga-Hama and Kumagai (*In*: Foreign Gene Expression in Fission Yeast: Schizosaccharomyces Pombe, Springer Verlag, ISBN 3540632700, 1997) and Guthrie and Fink (*In*: Guide to Yeast Genetics and Molecular and Cell Biology Academic Press, ISBN 0121822540, 2002).

A variety of suitable expression vectors, containing suitable promoters and regulatory sequences for expression in insect cells are well known in the art, and include, but are not limited to the pAC5 vector, the pDS47 vector, the pMT vector suite (Invitrogen) and the pIB vector suite (Invitrogen).

Furthermore, expression vectors comprising promoters and regulatory sequences for expression of polypeptides in plant cells are also well known in the art and include, for example, a promoter selected from the group, pSS, pB1121 (Clontech), pZ01502, and pPCV701 (Kuncz *et al*, *Proc. Natl. Acad. Sci. USA, 84* 131-135, 1987).

Expression vectors that contain suitable promoter sequences for expression in mammalian cells or mammals include, but are not limited to, the pcDNA vector suite supplied by Invitrogen, the pCI vector suite (Promega), the pCMV vector suite (Clontech), the pM vector (Clontech), the pSI vector (Promega), the VP16 vector (Clontech).

Methods of cloning DNA into nucleic acid vectors for expression of encoded polypeptides are well known in the art and are described for example in, Ausubel *et al* (*In*: Current Protocols in Molecular Biology. Wiley Interscience, ISBN 047 150338, 1987) or Sambrook *et al* (*In*: Molecular Cloning: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, New York, Third Edition 2001).

Following generation of a gene construct for expression of a labelled membrane transport protein, the gene construct must be introduced into the appropriate cell in order to analyze the translocation of the membrane transport protein. Methods of introducing the gene constructs into a cell or organism for expression are well known to those skilled in the art and are described for example, in Ausubel *et al* (In: Current Protocols in Molecular Biology. Wiley Interscience, ISBN 047 150338, 1987), (Sambrook *et al* (In: Molecular Cloning: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, New York, Third Edition 2001). The method chosen to introduce the gene construct in depends upon the cell type in which the gene

construct is to be expressed.

In one embodiment, the cellular host is a bacterial cell. Means for introducing recombinant DNA into bacterial cells include, but are not limited to electroporation or chemical transformation into cells previously treated to allow for said transformation.

In another embodiment, the cellular host is a yeast cell. Means for introducing recombinant DNA into yeast cells include a method chosen from the group consisting of electroporation, and PEG mediated transformation.

In another embodiment, the cellular host is a plant cell. Means for introducing recombinant DNA into plant cells include a method selected from the group consisting of *Agrobacterium* mediated transformation, electroporation of protoplasts, PEG mediated transformation of protoplasts, particle mediated bombardment of plant tissues, and microinjection of plant cells or protoplasts.

In yet another embodiment, the cellular host is an insect cell. Means for introducing recombinant DNA into plant cells include a method chosen from the group consisting of, infection with baculovirus and transfection mediated with liposomes such as by using cellfectin (Invitrogen).

In a preferred embodiment, the cellular host is a mammalian cell. Means for introducing recombinant DNA into mammalian cells include a means selected from the group comprising microinjection, transfection mediated by DEAE-dextran, transfection mediated by calcium phosphate, transfection mediated by liposomes such as by using Lipofectamine (Invitrogen) and/or cellfectin (Invitrogen), PEG mediated DNA uptake, electroporation, transduction by Adenoviuses, Herpesviruses, Togaviruses or Retroviruses and microparticle bombardment such as by using DNA-coated tungsten or gold particles (Agacetus Inc., WI,USA).

Accordingly, following introduction of the gene construct, membrane transport protein, or labelled membrane transport protein into a cell the amount of the membrane transport protein that has translocated to the plasma membrane is determined. Accordingly, a quantitative method of detecting a protein is preferred in order to determine the amount of the membrane transport protein that has translocated to the plasma membrane.

Standard solid-phase ELISA formats are particularly useful in determining the amount of a membrane transport protein that has translocated to the plasma membrane.

In one form such an assay involves immobilising or growing or incubating the cell onto a solid matrix, such as, for example a polystyrene or polycarbonate microwell or dipstick, a membrane, or a glass support (e.g. a glass slide). Preferably, the ELISA assay is performed upon the plate upon which the cells are grown.

An antibody or ligand that specifically binds the membrane transport protein or label is brought into direct contact with the cell, and forms a direct bond with any of the membrane transport protein or label present in said sample. This antibody is generally labelled with a detectable reporter molecule, such as for example, a fluorescent label (e.g. FITC or Texas Red) or an enzyme (e.g. horseradish peroxidase (HRP)), alkaline phosphatase (AP) or β-galactosidase, or alternatively a second labelled antibody can be used that binds to the first antibody. Following washing to remove any unbound antibody the label may be detected either directly, in the case of a fluorescent label, or through the addition of a substrate, such as for example hydrogen peroxide, TMB, or toluidine, or 5-bromo-4-chloro-3-indol-beta-D-galaotopyranoside (x-gal).

Following determining the amount of membrane transport protein that has translocated to the plasma membrane of a cell, the total amount of that membrane transport protein in the cell is determined using methods known in the art and/or described herein. Accordingly, comparison of the amount of membrane transport protein that has translocated to the plasma membrane to the total amount of that membrane transport protein in the cell provides a relative estimate of the amount of membrane transport protein that has translocated to the plasma membrane as a function of total membrane transport protein (for example as a percentage of total membrane transport protein). Such an estimate effectively "normalizes" the results of such an assay, reducing inter-assay variability and allowing comparisons between multiple assays.

In order to determine the total amount of membrane transport protein in a cell, the plasma membrane is permeabilized or disrupted to allow the detection means, e.g. a ligand or antibody, to enter the cell and bind the labelled membrane transport protein. In permeabilizing or disrupting a cell membrane it is important that the proteins within the cell are not degraded, and the membrane transport protein of interest that is

associated with the membrane (ie has translocated to the plasma membrane) is not degraded.

It is preferred that a cell is fixed prior to permeabilization/disruption, in order to stabilize and protect both the cell and the proteins contained within the cell. Preferably, the method of fixation does not disrupt or destroy the antigenicity of the membrane transport protein of interest, thus rendering it undetectable. Methods of fixing a cell are well known in the art and include for example, treatment with paraformaldehyde, treatment with alcohol, treatment with acetone, treatment with methanol, treatment with Bouin's fixative and treatment with glutaraldehyde.

In a particularly preferred embodiment, cells are fixed with paraformaldehyde prior to detecting a membrane transport protein at the plasma membrane.

Following fixation, cells are permeabilised using a method well known in the art, such as, for example, treatment with saponin, treatment with n-octyl-ßD-glucopyranoside, treatment with deoxycholate, or treatment with a non-ionic detergent such as Triton X-100 or NP-40 detergents, such as, for example CHAPS are also useful in this respect, and low concentrations of ionic detergents, such as, for example SDS are also of use. Alternatively, a fixed or unfixed cell may be permeabilized with a peptide, such as, for example, streptolysin-O (SLO) or mellitin (Katsu *et al*, *Biochim. Biophys. Acta. 939*:57-63, 1988). Alternatively, extraction using organic solvents, such as, for example, methanol or a combination of methanol and acetone may also be used.

In a particularly preferred embodiment, the cells are permeabilized with saponin.

Following permeabilization of a cell the total amount of the membrane transport protein in said cell is detected using methods well known in the art and/or described herein.

Comparison of the amount of the membrane transport protein of interest that has translocated to the plasma membrane to the total amount of the membrane transport protein in a cell provides an estimation of the relative amount of the membrane transport protein of interest that was translocated to the plasma membrane. Accordingly, the amount of membrane transport protein of interest that has translocated to the cell membrane is determined as a function of the total amount of the membrane transport protein in a cell. Such an estimate results in a normalization of the result of

an assay, reducing the effect of any variation in the level (eg expression level) of the membrane transport protein. This normalization enables the direct comparison of results of different assays.

As will be apparent to the skilled artisan, the present method also provides means to monitor or determine the turnover (or recycling) of a membrane transport protein in a plasma membrane. Accordingly, a further embodiment of the invention relates to a method of determining the amount of recycling of a membrane transport protein, the method comprising determining the amount of a labelled membrane transport protein that has been translocated to the plasma membrane of a cell at various points in time and/or following exposure of said cell to a condition that may affect the recycling of said labelled membrane transport protein.

Using the method of the present invention, the amount of a membrane transport protein that has translocated to the plasma membrane is determined. The process of determining the amount of membrane transport protein that has translocated to the plasma membrane is then repeated at various time points. By comparing the amount of membrane transport protein that has been translocated to the plasma membrane at various time points, the amount of recycling or turnover rate of the membrane transport protein is determined.

Accordingly, a reduction in the amount of membrane transport protein that is detected at the plasma membrane, with little or no associated change in the total membrane transport protein in the cell, suggests that the membrane transport protein is being recycled, or translocated from the membrane to an intracellular location. Alternatively, an increase in the amount of a membrane transport protein that is detected at the cell membrane, with little or no associated change in the total membrane transport protein in the cell, suggests an increase in membrane transport protein translocation to the plasma membrane.

As stated previously, such a method is also of use in determining conditions that affect translocation and/or membrane transport protein recycling. For example, previous research has shown that temperature effects the translocation and recycling of some membrane transport proteins, such as, for example, mutant forms of CFTR (Cheng *et al, Cell, 63(4)*, 827-834, 1990). Accordingly, the amount of translocation and/or recycling of this membrane transport protein may be determined using the method of

the present invention by detecting the amount of labelled mutant CFTR at the cell membrane at various temperatures. Accordingly, cells are incubated at various temperatures, and the amount of membrane transport protein that has translocated to the plasma membrane of said cell is determined. As these results are normalized, they may be directly compared. Accordingly, any change in the relative mount of a membrane transport protein that is translocated to the plasma membrane is an effect of the change in temperature.

Furthermore, this method may be used to detect the rate of translocation and/or recycling following a change in temperature.

As will be apparent to the skilled artisan, the method of the present invention may be used to detect the amount of more than one membrane transport protein that has translocated to the plasma membrane of a cell. In adapting this method to determine the amount of two or more membrane transport proteins that have translocated to the plasma membrane of the cell, each of these membrane transport proteins should be labelled with a different label. Alternatively, detection of the extracellular domain of each of these membrane transport proteins using a different ligand that is detectable with a different means (i.e. each are labelled with a fluorophore that is excitable at the wavelength different to the other label) will differentiate between the membrane transport proteins. This facilitates the specific detection of each of the membrane transport proteins of interest.

As will be apparent to the skilled artisan the methods described herein relating to determining the amount of a membrane transport protein that has translocated to the plasma membrane of a cell may be modified to detect the effect of, for example a mutation in the membrane transport protein, or to determine a mutation that modulates the translocation of said membrane transport protein to the cell membrane.

As used herein, the term "modulates" shall be taken to mean either an increase or decrease in the translocation of a membrane transport protein to the plasma membrane. A decrease in the amount of membrane transport protein that is translocated to the plasma membrane of a cell may be the result of increased recycling (as may be determined using methods described herein), or, alternatively, the mutation may result in a reduced translocation of the membrane transport protein.

Another aspect of the present invention provides a method of determining a mutation in a nucleic acid encoding a membrane transport protein that modulates the amount of the membrane transport protein. Such a method involves detecting the amount of a mutant membrane transport protein that translocates to the plasma membrane in a cell and detecting the amount of a labelled wild type membrane transport protein that translocates to the plasma membrane.

A result of such an assay that indicates an increase in the amount of translocation of the labelled mutant membrane transport protein to the cell membrane compared to the control membrane transport protein, indicates that the mutation causes an increase in the translocation of that membrane transport protein.

A result of such an assay that indicates a decrease in the amount of translocation of the labelled mutant membrane transport protein to the cell membrane compared to the control membrane transport protein, indicates that the mutant membrane transport protein causes either a reduction in the translocation of that membrane transport protein, or alternatively an increase in the recycling of said membrane transport protein.

The nucleic acid encoding the mutant membrane transport protein of use in this aspect of the present invention may be derived from a subject, for example, a subject that is suffering from a disorder that may be caused by the increased or decreased translocation of a membrane transport protein to the plasma membrane. Alternatively, the nucleic acid encoding the mutant membrane transport protein of use in this aspect of the present invention may be generated using methods well known in the art and described, for example, in Ausubel *et al* (In: Current Protocols in Molecular Biology. Wiley Interscience, ISBN 047 150338, 1987), (Sambrook *et al* (In: Molecular Cloning: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, New York, Third Edition 2001). For example, a membrane transport protein may be mutated using mutagenic PCR (Dieffenbach (ed) and Dveksler (ed) (*In*: PCR Primer: A Laboratory Manual, Cold Spring Harbour Laboratories, NY, 1995), or by site-direscted mutagenesis.

A further aspect of the present invention provides a method of determining an agent that modulates the amount of a membrane transport protein translocated to the plasma membrane of a cell. This method comprises the use of any of the previously described methods to determine the amount of a labelled membrane transport protein translocated

to the plasma membrane in a cell exposed to an agent and in a cell that is not exposed to an agent. Accordingly, this assay provides the means to determine an agent that modulates the translocation of a membrane transport protein.

In one embodiment, the method of determining the amount of a protein translocated to the plasma membrane of a cell, said said method comprising:

(a) Performing a method of the present invention to determine translocation of a wild-type or mutant form of the membrane trnasport protein in the presence of a candidate agent; and

(b) comparing translocation of the membrane transport protein at (a) to translocation of the wild-type or mutant membrane transport protein in the absence of the candidate agent, wherein a difference in translocation indicates that the candidate agent modulates translocation of the membrane transport protein.

In a preferred embodiment, this aspect of the present invention provides a method of determining an agent that modulates translocation of a membrane transport protein to the plasma membrane of a clel said method comprising:

(a) performing a method of the present invention to determine translocation of a wild-type or mutant form of the membrane transport protein in the absence of a candidate agent;

(b) performing a method of the present invention to determine translocation of a wild-type or mutant form of the membrane transport protein in the prescence of the candidate agent; and

(c) comparing translocation of the membrane transport protein at (a) and (b) wherein a difference in translocation indicates that the candidate agent modulates translocation of the membrane transport protein.

In an alternative or additional embodiment, a control cell (that is the cell to which the cell exposed to the agent is compared) is one that has not been infected with a retrovirus comprising the nucleic acid encoding the membrane transport protein of interest. Accordingly, such a cell does not express the recombinant membrane transport protein of interest. Such a cell represents a negative control, as little or no (or at least only background levels) of the membrane transport protein are detected.

In a further alternative or additional embodiment, a control for an assay testing an agent that modulates the translocation of a membrane transport protein comprises a ligand of

a membrane transport protein not expressed by the cell. Such a control determines whether or not the ligand/s may be binding to target proteins other than that to which they are targeted.

In a still further preferred embodiment, a control for an assay testing an agent that modulates the translocation of a membrane transport protein comprises transforming a cell with an additional protein, preferably an additional membrane transport protein that is known to translocate to the membrane and be recycled. Preferably, the membrane transport protein is translocated and recycled by a mechanism (i.e. through a pathway) different to the membrane transport protein of interest. Detection of this membrane transport protein especially when translocation has been induced serves as a positive control, in that it determines whether or not the membrane transport protein/s have been successfully translocated and/or successfully detected.

As used herein the term "agent" shall be taken in its broadest context to include any chemical compound, polynucleotide, peptide, polypeptide, protein, lipid, carbohydrate, small molecule, natural product, polymer, etc. that is able to bind selectively and stoichiometrically, whether covalently or not, to one or more specific sites on a membrane transport protein. Preferably, the agent modulates the translocation of said membrane transport protein. The agent may bind to its target via any means including hydrophobic interactions, hydrogen bonding, electrostatic interactions, van der Waals interactions, pi stacking, covalent bonding, or magnetic interactions amongst others.

A result of such an assay that indicates an increase in the translocation of a labelled membrane transport protein to a plasma membrane in a cell exposed to an agent compared to the amount detected in a cell that is not exposed to an agent suggests that said agent increases the translocation of said membrane transport protein.

A result indicating a decrease in the translocation of a labelled membrane transport protein to a plasma membrane in a cell exposed to an agent compared to the amount detected in a cell that is not exposed to an agent suggests that said agent either decreases the translocation of said membrane transport protein or increases the recycling of said membrane transport protein. Accordingly, further testing utilizing a time course experiment described herein may be required. For example, the amount of membrane transport protein translocated to the membrane of a cell may be detected at various time points following the exposure of a cell to an agent. If the amount of the

membrane transport protein translocated to the plasma membrane rapidly decreases after the addition of an agent, the agent is likely to increase the recycling of the membrane transport protein.

Such an assay is of particular use in determining agents that may be of use in the treatment of a disease that is associated with increased or decreased translocation of a membrane transport protein to the plasma membrane.

For example, studies have suggested that GLUT4 is not effectively translocated to the plasma membrane of a cell in subjects suffering from type II diabetes mellitus. Accordingly, a library of potential agents, such as, for example, a small molecule library, may be screened using the method of the present invention to determine the molecule/s that increase the translocation of GLUT4 to the plasma membrane. One advantage of the present invention resides in the normalization of the results attained for each of the molecules tested. Accordingly, only one control sample (ie a sample that is cultured under the same conditions as the test samples with the exception of the test agent) is required for the entire screen. Each of the test samples may then be compared to both the control sample and to each other to determine those agents that increase translocation of GLUT4 to the plasma membrane. Additionally, as this test system may be performed in, for example, a 96 or 384 well tissue culture plates, it is clearly amenable to high throughput screening of agents that modify translocation of a membrane transport protein.

In one embodiment, the agent is isolated from a random peptide library. A random peptide library random peptide may be generated and screened as described in U.S. Patent Application No. 5,733,731, 5,591,646 and 5,834,318. Generally such libraries are generated from short random oligonucleotides that are expressed *in vitro*. Such oligonucleotides may be expressed by the cell in which the assay is performed, or alternatively the peptide may be produced by any other means known to the person skilled in the art (such as, for example, by *in vitro* translation, by expression and affinity purification, by phage display, retroviral display, bacterial surface display, bacterial flagellar display, bacterial spore display, yeast surface display, mammalian surface display, and methods of *in vitro* display including, mRNA display, ribosome display and covalent display) and subsequently cultured with the cell in which the assay is performed.

A chemical small molecule library is also clearly contemplated for the identification of an agent that modulate translocation of a membrane transport protein to the plasma membrane. Chemical small molecule libraries are available commercially or alternatively may be generated using methods well known in the art, such as, for example, those described in U.S. Patent No. 5,463,564.

In a preferred embodiment, not only does the agent modulate that translocation of a membrane transport protein of interest, but it also modulates the activity of that membrane transport protein. Accordingly, the activity of a membrane transport protein that is only active at the plasma membrane, for example, an ion channel, may be modulated by such an agent. An agent that reduced the translocation of such a membrane transport protein to the plasma membrane would also reduce the activity of said membrane transport protein, while an agent that induced the translocation of such a membrane transport protein to the plasma membrane would also induce the activity of the membrane transport protein. Accordingly, this assay is of particular use in determining agents that a putative therapeutics for the treatment for a wide variety of disorders that are associated with altered translocation of membrane transport proteins to the plasma membrane.

The present invention is further described with reference to the following non-limiting examples

EXAMPLE 1

GENERATION AND EXPRESSION OF A LABELLED GLUT4 PROTEIN

Using standard techniques in the art, as described in, for example, in Ausubel *et al* (*In*: Current Protocols in Molecular Biology. Wiley Interscience, ISBN 047 150338, 1987) or Sambrook *et al* (*In*: Molecular Cloning: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, New York, Third Edition 2001), a nucleic acid encoding GLUT4 was generated, comprising a HA tag in the first extracellular domain of the protein essentially as described in Quon, et al. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 5587-5591 (nucleic acid represented in SEQ ID NO: 4 and protein represented in SEQ ID NO: 3). This gene construct was inserted into the vector pBABE (Pear, et al. (1993) Proc. Natl Acad. Sci. U.S.A. 90, A diagramatic representation of the protein encoded protein is shown in Figure 1A.

Additional gene constructs were also generated comprising a nucleic acid encoding mutant forms of GLUT4 were generated (these constructs encoded the TAIL mutant of GLUT4 (SEQ ID NO: 5), the L489,490A mutant of GLUT4 (SEQ ID NO: 6) and the F5A mutant of GLUT4 (SEQ NO: 7), each tagged with a HA tag), comprising a HA tag in the first extracellular domain of the protein, essentially as described in Piper *et al, The Journal of Cell Biology, 121(6)*:1221-1232, 1993, Marsh *et al, JCB, 130(5)*: 1081-1091, 1995, Shewan *et al. Biochem. J, 350:* 99-107, 2000 and Shewan *et a, Mol. Biol. Of Cell, 14*: 973-986, 2003. The proteins encoded by these nucleic acids are represented in Figure 1B.

Following generation of these gene constructs, they were transduced into 3T3-L1 fibroblasts using a retroviral transduction system. The details of this transduction system are described in detail in Shewan et al Biochem J 350: 99-107, 2000. These fibroblast cells may be differentiated into adipocytes, facilitating the stuidy of GLUT4 translocation in both fibroblasts and adipocytes.

The subcellular localization of the expressed proteins in both fibroblasts and adipocytes was determined using immunofluorescence (as shown in Figure 3). Interestingly, in fibroblasts, but not adipocytes, GLUT4 traverses the plasma membrane continuously in the absence of insulin.

EXAMPLE 2

GENERATION OF AN ASSAY TO DETERMINE THE LOCALIZATION OF GLUT4

Retrovirally-transduced fibroblasts expressing HA-tagged GLUT 4 were differentiated into adipocytes. These adipocytes were then subcultured for 30 hours, after which media was removed from a sample of these adipocytes and replaced with media containing 200 nM insulin. Following incubation for an adequate period of time cells were fixed with paraformaldehyde. The HA epitope incorporated into GLUT4 was then detected using an anti-HA monoclonal antibody (Covance, Berkeley, CA 94710). Cell membranes were then permeabilized with 0.1% saponin, and the anti-HA antibody detected with a ALEXA-488-conjugated goat-anti-mouse antibody (Molecular Probes, Eugene, OR 97402). Fluorescence was then detected with a multi-well plate reader (BMG Technologies GmBH, 77656 Offenburg) at 485 nm exc and 520 nm emm. This method detects the amount of GLUT4 that has been translocated to the plasma membrane.

Another sample of adipocytes was fixed and the membranes permeabilized using 0.1% saponin. The HA epitope incorporated into GLUT4 is then detected using an anti-HA monoclonal antibody (Covance), and the anti-HA antibody detected with ALEXA-488-conjugated goat-anti-mouse antibody (Molecular Probes). Fluorescence was then detected with a multi-well plate reader (BMG Technologies) at 485 nm exc and 520 nm emm. This method detects the amount of GLUT4 in the cell. (An overview of this method is shown in Figure 2).

As shown in Figure 4B insulin causes a rapid increase in the amount of GLUT4 that is translocated to the membrane, while this effect is not observed in other proteins. The amount of GLUT4 translocated to the plasma membrane was then determined relative to the total labelled GLUT4 as determined using the above-described method. As shown in Figure 4C 200 nM insulin rapidly induced approximately 35% of the GLUT4 in a cell to be translocated to the plasma membrane (ie within 10 minutes of the addition of insulin).

Furthermore, experiments using various concentrations of insulin showed that the effect of insulin on translocation of GLUT4 was dose responsive, with any effect of insulin abrogated by the addition of wortmannin (Figure 4D).

The various mutant forms of GLUT4 were also transduced into 3T3-L1 fibroblast cells and 3T3-L1 adipocytes. As shown in Figures 5A and 5B GLUT4 was translocated to the plasma membrane in both fibroblasts and adipocytes. The GLUT4-TAIL mutant, which is only observed in the early/recycling endosomal compartments was found to be as effective in translocating as wild-type GLUT4. The L489,490A and F5A mutants, which are believed to be impaired in their internalization/recycling show a clear increase in translocation in adipocytes. Accordingly, these mutants represent good models for determining agents that produce moderate affects on GLUT4 translocation.

EXAMPLE 3
AN ASSAY TO DETERMINE AN AGENT THAT AFFECTS THE TRANSLOCATION OF GLUT4

Retrovirally-transduced fibroblasts expressing HA-tagged GLUT4 are differentiated into adipocytes, which are, in turn, subcultured in coated 96 well plates for 30 hours. Following this time media is removed for two hours and then replaced with media supplemented with insulin or a small molecule isolated from a small molecule library. Each of these small molecules (or insulin) are replica plated in order to provide two wells of cells that have received the same treatment. Cells are then incubated before being fixed and analysed using the method described in Example 2.

Samples are then analysed to determine those small molecules that are capable of inducing the same degree of GLUT4 translocation as is observed for insulin.

EXAMPLE 4
AN ASSAY TO DETERMINE AN AGENT THAT SUPPRESSES THE TRANSLOCATION OF GLUT4

Retrovirally-transduced fibroblasts expressing HA-tagged GLUT4 are differentiated into adipocytes, which are, in turn, subcultured in coated 96 well plates for 30 hours. Following this time media is removed for two hours and then replaced with media supplemented with both insulin and a peptide isolated from a random peptide library. Each of these random peptides are replica plated in order to provide two wells of cells that have received the same treatment. Cells are then incubated before being fixed and analysed using the method described in Example 2.

Samples are then analysed to determine those peptides that are capable of suppressing GLUT4 translocation in the presence of insulin.

EXAMPLE 5

AN ASSAY TO DETERMINE AN AGENT THAT INDUCES TRANSLOCATION OF THE CFTR

A gene construct encoding the Δ508 mutation of the CFTR (SEQ ID NO: 24) is modified to incorporate three consecutive c-myc tags in the first predicted extracellular domain. Murine lung epithelial cells are then retrovirally transduced with this gene construct and said modified protein analyzed to determine the cellular localization of the mutant CFTR.

Cells are cultured for approximately 30 hours before media is removed and replaced with media supplemented with a peptide isolated from a random peptide library. Each of these random peptides are replica plated in order to provide two wells of cells that have received the same treatment. Cells are then incubated before being fixed and analysed using the method essentially as described in described in Example 2, however the antibody used to detect the labelled CFTR is a monoclonal anti-c-myc antibody (Sigma-Aldrich, Sydney, Australia).

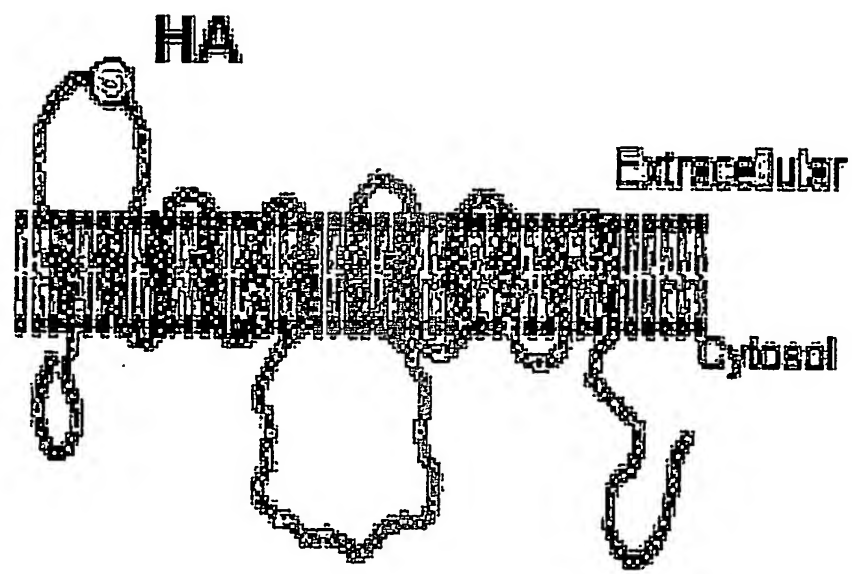


Figure 1A

A

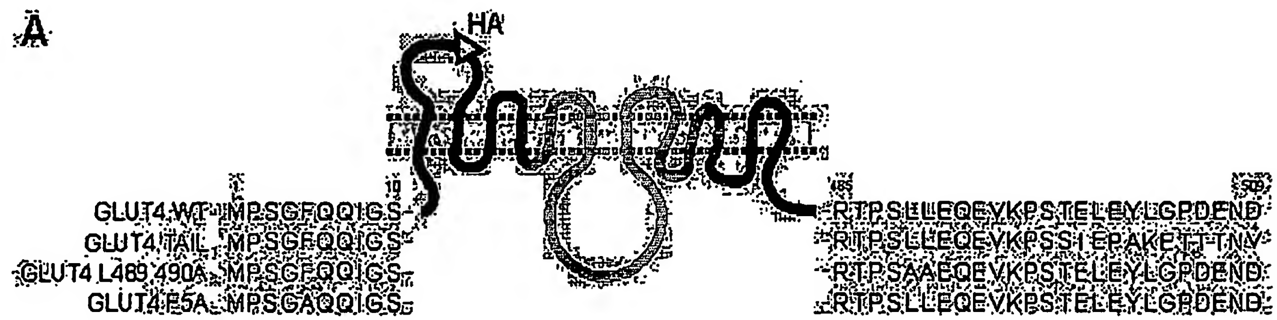

HA
1
10
489
509
GLUT4 WT MPSGFQQIGS
GLUT4 TAIL MPSGFQQIGS
GLUT4 L489 490A MPSGFQQIGS
GLUT4 F5A MPSGAQQIGS
RTPSLLEQEVKPSTELEYLGPDEND
RTPSLLEQEVKPSSIEPAKETTTNV
RTPSAAEQEVKPSTELEYLGPDEND
RTPSLLEQEVKPSTELEYLGPDEND

Figure 1B

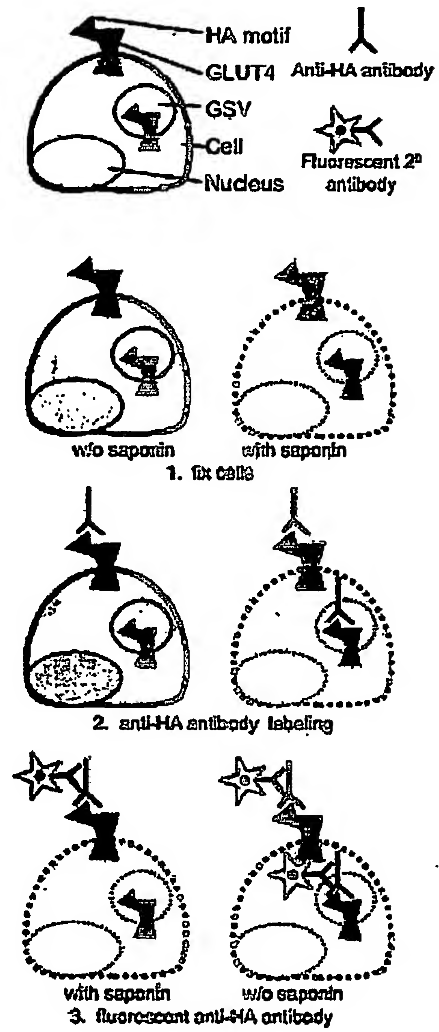

HA motif
GLUT4
GSV
Cell
Nucleus
Anti-HA antibody
Fluorescent 2° antibody
w/o saponin
with saponin
1. fix cells
2. anti-HA antibody labeling
with saponin
w/o saponin
3. fluorescent anti-HA antibody

Figure 2

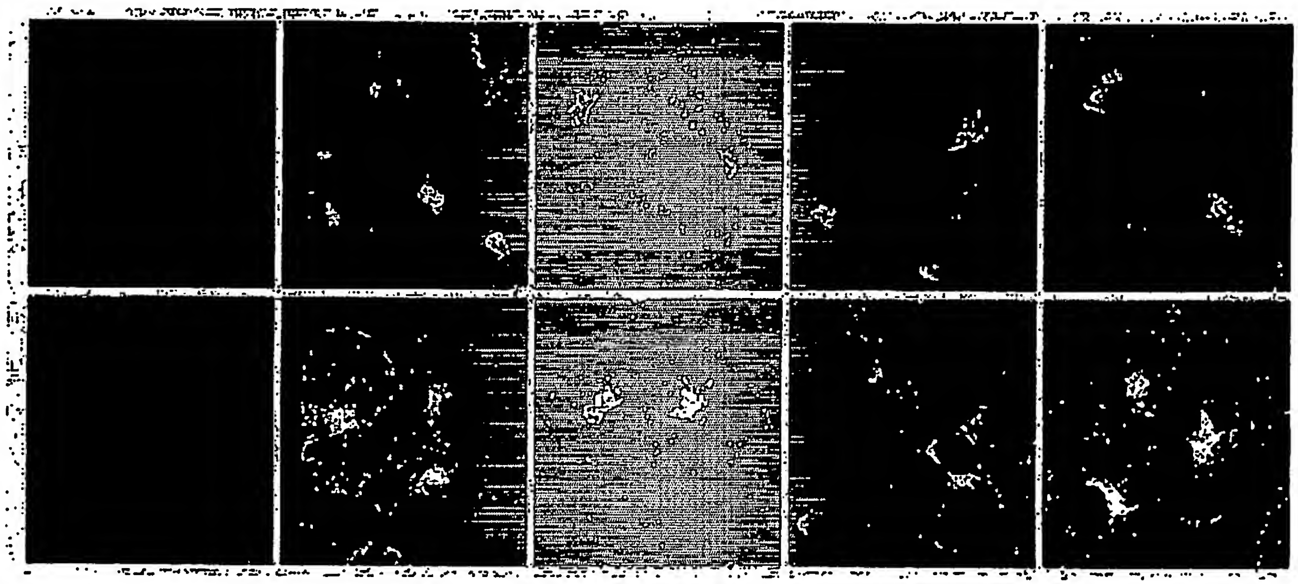
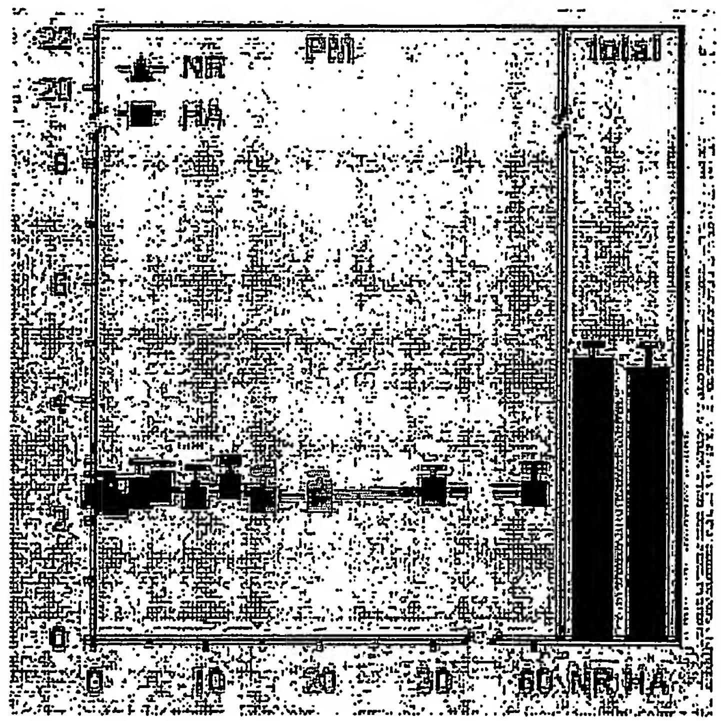


Figure 3



time (min)

Figure 4A

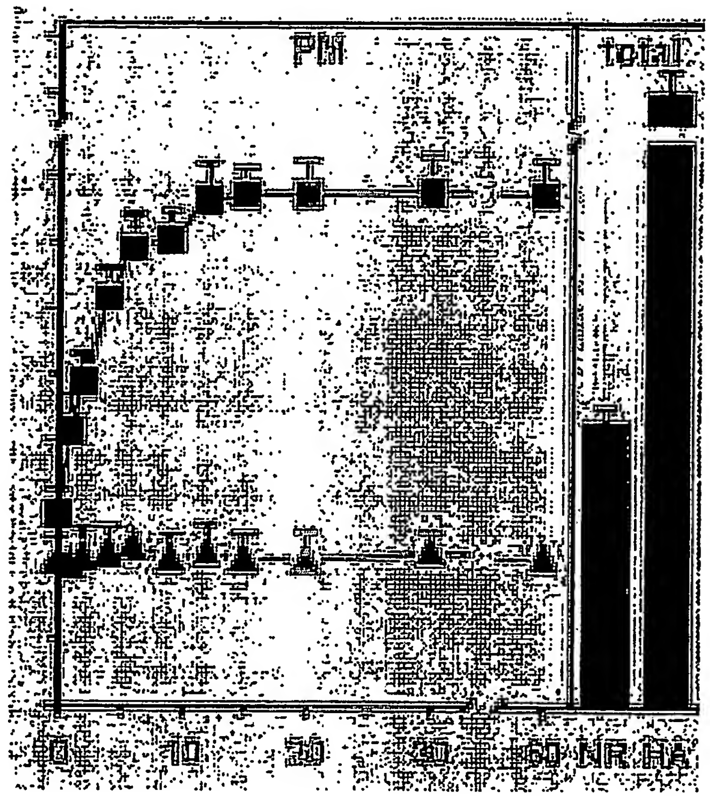


Figure 4B

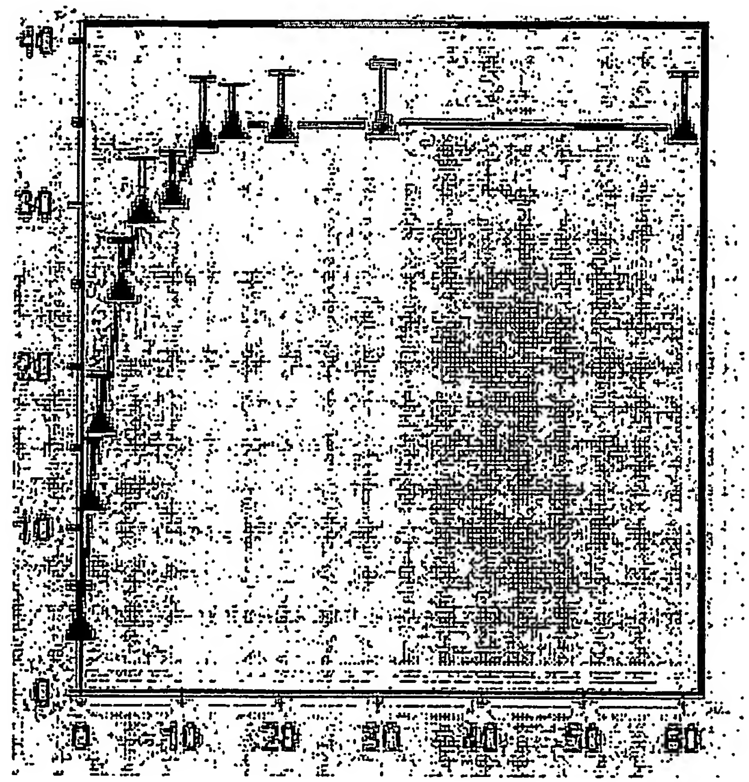
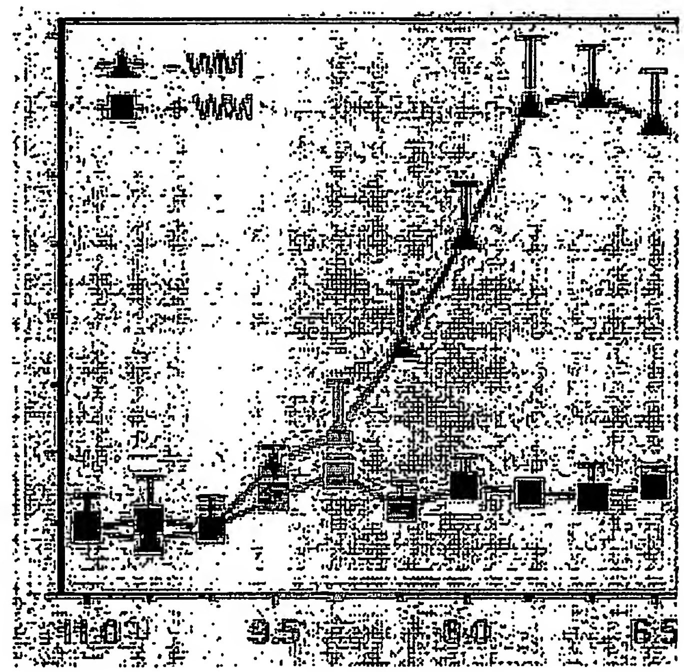


Figure 4C



time (min)

Figure 4D

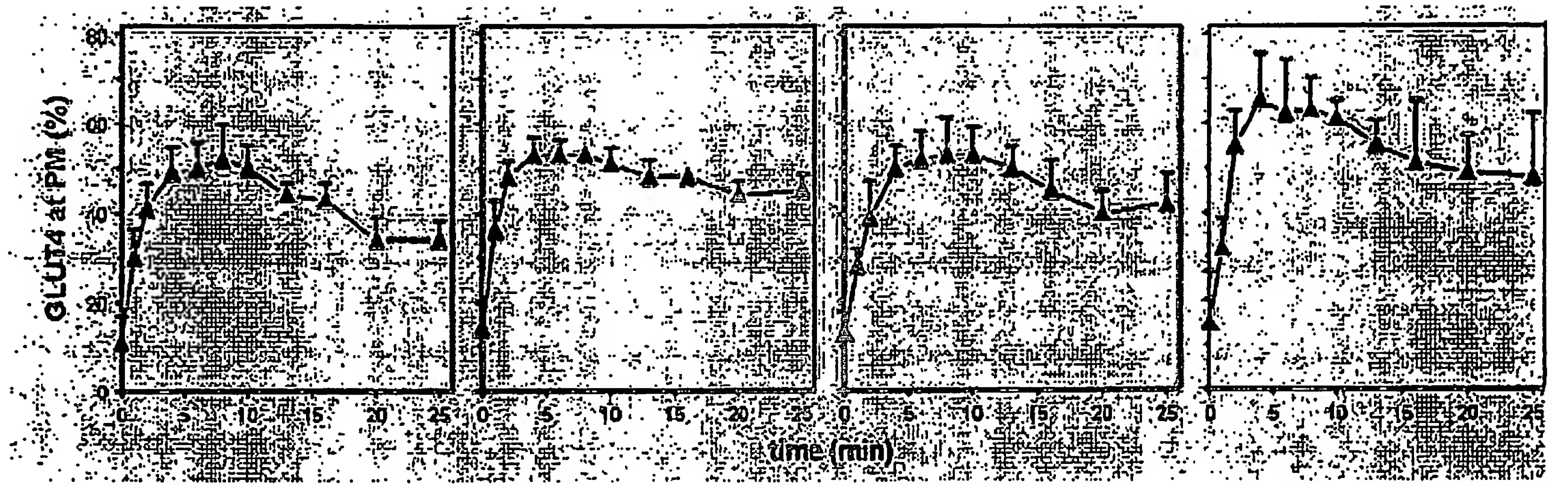


Figure 5A

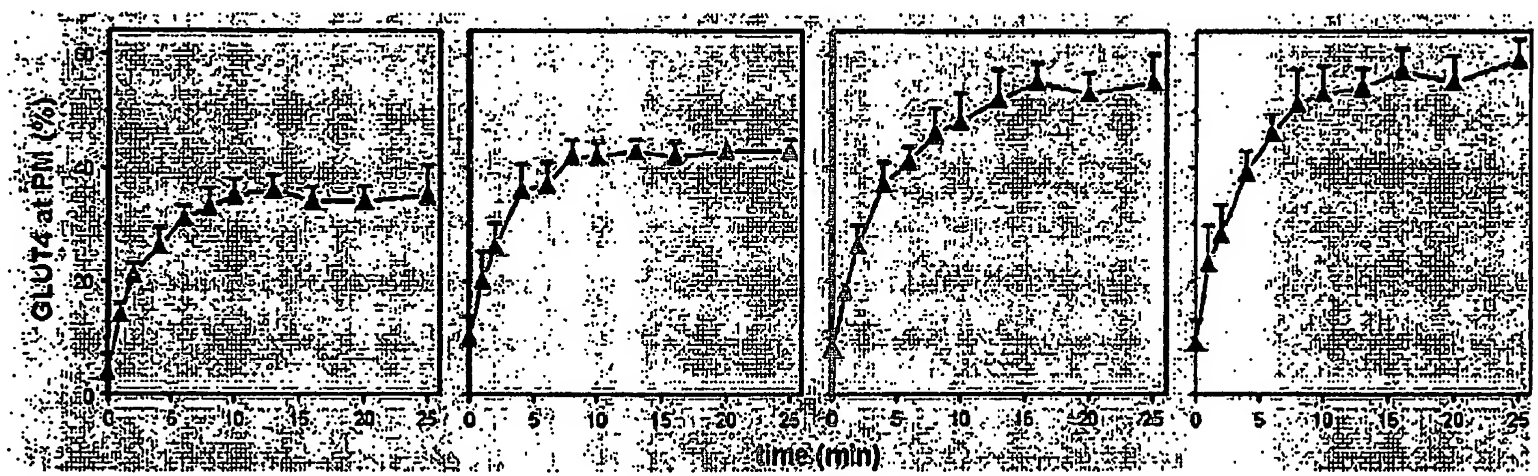


Figure 5B